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Project Title: Modulation of the synthesis of the main preformed antifungal compound as a basis for the prevention of postharvest disease of *C. gloeosporioides* in avocado fruits

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Fruit resistance, quiescent infections, Fruit diseases

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Modulation of the synthesis of the main preformed antifungal compound as a basis for the prevention of postharvest disease of *Colletotrichum gloeosporioides* in avocado fruits

The most important pathological factor limiting fruit life after harvest in subtropical fruits are quiescent infections of anthracnose caused by *Colletotrichum gloeosporioides*. Prusky and Keen elucidated the mechanism of resistance in avocado fruits to quiescent infections of *C. gloeosporioides* and determined that the major biocide involved is the preformed compound,1-acetoxy-2-hydroxy-4-oxo-heneicosa-13,15 diene.

Two possibilities exist for maintaining fungitoxic levels of antifungal compounds in the tissue of ripening fruits: (i). Prevention of catabolism (ii). Induction of synthesis. Previous work has demonstrated that increased fruit susceptibility after fruit harvest occurs through diene catabolism mediated by oxidation of the antifungal compound by the enzyme lipoxygenase. Levels of a non-specific inhibitor, epicatechin, in turn, regulate activity of lipoxygenase, present in the peel of unripe but not ripe fruit. In this proposal, we examined the possibility of exploiting induced synthesis of the antifungal compound for the study of the synthetic pathway.

The general objective of the present research was to study the mechanism of biosynthesis of natural antifungal compounds in order to regulate the process of resistance to postharvest diseases in ripening avocado fruits. The specific objectives of the research were:

1. To localize synthesis of the antifungal diene and modulate the process by biotic or a biotic elicitors.
2. To determine the relation between synthesis of the diene and accumulation in the peel and fruit resistance to decay
3. To characterize the biosynthetic pathway and the diene and the genes involved.

The analysis of the antifungal compounds in avocado resulted in the detection of a new antifungal compound (E, Z, Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5, 12,15-triene. This new compound was shown to inhibit spore germination of *C. gloeosporioides* similarly as the antifungal diene. We had localized one of the biosynthetic places of these antifungal compounds in specialized idioblast cells (oil cells) in the mesocarp that can be easily enhanced by elicitors as ethylene. Results have also suggested that the antifungal compounds can be “exported” from the mesocarp to the pericarp where its main activity takes place. The search for the biosynthesis of antifungal compounds and the genes involved took two directions i. direct search for specific genes involved in the synthesis of the diene and ii. Indirect selection of genes using the differential display library. We have cloned Δ^9 and Δ^{12} desaturase, a protein kinase and a elongase that their transcriptional activation is significantly enhanced during the enhanced synthesis of the antifungal diene. Although we are far away from a complete elucidation of the synthesis of the antifungal compound we have stepped forward determining some of the key steps that might be involved in its synthesis.

Achievements:

The main significant achievements of the present project are:

1. Elucidation of a new preformed antifungal compound in avocado fruits
2. Localization of the antifungal compounds and characterization of the mechanism of elicitation of the compounds by abiotic elicitors.
3. Cloning of the Δ^9-stearoyl ACP desaturase, Δ^{12}-Fatty Acid Desaturase, Serine/threonine Kinase, and a fatty acid elongase from avocado fruit.
4. Characterization of the expression of Δ^9-stearoyl ACP desaturase during biotic and abiotic stresses affecting the antifungal diene.

1.**Elucidation of a new preformed antifungal compound in avocado fruits**

A new compound was isolated from avocado, *Persea americana* Mill., idioblast cell oil. Its structure was determined as (E, Z, Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5, 12,15-triene. This new compound was shown to inhibit spore germination of the fungal pathogen *C. gloeosporioides*. The full characterization of other compounds that have been previously partially described is also reported.

2. **Localization of the antifungal compounds and characterization of the mechanism of elicitation of the compounds by abiotic elicitors**.

It has previously been demonstrated that exposure of whole avocado fruits cv. Fuerte to 40 μl/l ethylene for 3 h enhances the level of antifungal 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene in the fruit pericarp. Exposure of 1-2 mm slices of fruit pericarp and mesocarp to ethylene enhanced the level of the antifungal diene in the mesocarp only. Since most of the antifungal diene in the mesocarp is compartmentalized in idioblast the effect of ethylene was tested on isolated idioblast. Exposure of idioblasts to ethylene increased the level of antifungal diene twofold within 3 hs. This effect was temperature dependent. Three hours exposure of idioblast to ethylene at 35^0C doubled the diene content compared to less than 50% increase after three hours at 20^0C. Incubation of idioblast with [2-^{14}C] malonyl- CoA or [1-^{14}C] acetate in the presence of ethylene, showed the incorporation of the label

into a compound that co-chromatographed with the antifungal diene. The compound induced by ethylene and released from the cells was identified by NMR as the antifungal diene. The present report suggests that ethylene can induce the synthesis of the antifungal diene in idioblast and the export of this compound to the pericarp of the fruits.

3. Characterization of the expression of Δ^9-stearoyl ACP desaturase during biotic and abiotic stresses affecting the antifungal diene.

The expression of the avocado homologue PAD1 encoding Δ^9-stearoyl ACP desaturase was enhanced by multiple stimuli - inoculation with *Colletotrichum gloeosporioides*, exposure to ethylene or CO_2, or low temperature (4 ^oC) and fruit wounding. This enhanced expression was correlated with an increase in the preformed antifungal (Z, Z)-1-acetoxy-2- hydroxy- 4-oxo- heneicosa-12, 15-diene. Treatments affecting up regulation of PAD1 increased as well the concentration of 18:2 fatty acid and the incorporation of C^{14}- linoleate to the antifungal diene. PAD1 expression was however down- regulated by light. Fruits with enhanced Δ^9-stearoyl ACP desaturase expression were more resistant to *C. gloeosporioides.* It is suggested that the enhanced Δ^9-stearoyl ACP desaturase expression which increases the concentration of unsaturated fatty acids, affects the synthesis of the This would result in the increase synthesis of the antifungal diene compound and the consequent enhanced resistance to fungal attack.

4. Cloning of the Δ^9-stearoyl ACP desaturase, Δ^{12}-Fatty Acid Desaturase, Serine/threonine Kinase, and a fatty acid elongase from avocado fruit.

As part of the effort to understand the possible mechanism of biosynthesis of the antifungal diene genes encoding for proteins at different points at the biosynthetic pathway were cloned. The characterization of these genes and the dynamics of their expression will be studied during the PhD student thesis of Xuejun Wang.

Cooperation:

A significant cooperation was kept since the early stages of the project through e-mails between all the participants. Dr. Fred Domergue, Post Doct working in Dr. Browse laboratory was in constant contact with the Israeli PI. As part of this interaction Dr. Prusky spent more than 2 months at the Institute of Biological

Chemistry. Washington State University at Pullman. During that period he was able to develop different aspects of the project in joint collaboration with Dr. Browse. Short visits took place by the PI in both Riverside California and Pullman, Washington, as part of the consulting and collaborations to solve specific problems.

List of publications

Reviewed papers:

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Ethylene enhances the antifungal lipid content in idioblast cells from avocado mesocarp.
Phytochemistry 49: 2291-2298.

Domergue, F, Helms, G.H., Prusky, D., and Browse, J. 2000.
Antifungal compounds from idioblast cells isolated from avocado fruits.
Phytochemistry 54:183-189.

Madi, L. and Prusky, D. 1999.
Sequence of a cDNA clone encoding an avocado (*Persea americana*) Δ^9 stearoyl-acyl carrier protein desaturase. Plant Physiol. 121:1054.

Book Chapters:

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Abstracts:

Leikin-Frenkel, A., Ardi, R., Kobiler, I. and Prusky D. 1997. Effect of ethylene treatments on the levels of antifungal compounds in oil cells of avocado mesocarp. Phytoparasitica 25:262

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2. SUBMITTED PAPERS

Plant Molecular Biology

Light and stress regulate Δ^9-stearoyl ACP desaturase expression, fatty acids composition, antifungal diene level and resistance to fungal attack

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Abstract

The expression of the avocado homologue PAD1 encoding Δ^{9}-stearoyl ACP desaturase was enhanced by multiple stimuli - inoculation with *Colletotrichum gloeosporioides*, exposure to ethylene or CO_2, or low temperature (4 ^{o}C) and fruit wounding. This enhanced expression was correlated with an increase in the preformed antifungal (Z, Z)-1-acetoxy-2- hydroxy- 4-oxo- heneicosa-12,15-diene. Treatments affecting up regulation of PAD1 increased as well the concentration of 18:2 fatty acid and the incorporation of C^{14}- linoleate to the antifungal diene. PAD1 expression was however down- regulated by light. Fruits with enhanced Δ^{9}-stearoyl ACP desaturase expression were more resistant to *C. gloeosporioides.* It is suggested that the enhanced Δ^{9}-stearoyl ACP desaturase expression that increases the concentration of unsaturated fatty acids, affects the synthesis of the antifungal diene compound and the consequent enhanced resistance to fungal attack.

Introduction

One of the primary products of fatty acid synthesis are palmitoyl-acyl-carrier (16:0-ACP) and D^{9} stearoyl -ACP (18:0-ACP). The soluble plastid enzyme, stearoyl-ACP desaturase (EC1.14.99.6), introduces the first double bond into stearoyl-ACP, between carbons 9 and 10, to produce oleoyl-ACP (18:1 Δ^{9}-ACP). Additional double bonds are introduced into oleic acid or palmitic acid by Δ^{12} desaturases after incorporation of the fatty acids into lipids. Δ^{9} stearoyl-ACP desaturase activity is a key determinant of the overall level of fatty acid desaturation (Thompson *et al*., 1991; Shanklin and Somerville, 1992).

In *Arabidopsis thaliana,* seven loci have been identified as fatty acid desaturase-encoding (Yadav *et al*., 1993; McConn *et al*., 1994; Ardi *et al*., 1998). Of these, three loci- *fad3, fad7* and *fad8* are responsible for the production of trienoic fatty acids by unsaturation at the ω-3 position. Introduction of the *A. thaliana fad7 g*ene into tobacco under the control of a constitutive promoter resulted in a significant elevation of trienoic levels (Kodama *et al*., 1994).

Plant desaturases respond to different environmental and physical stresses. Low temperature increases transcription of desaturase in maize, tobacco, arabidopsis and soybean (Cheesbrough, 1990; Miquel *et al*., 1993; Somerville, 1995; Yong Moon *et*

al., 1995; Ishizaki-Nishizawa *et al*., 1996). Light has also been reported as a stimulus that inducing desaturases (*fad7*) in *A. thaliana*. In wheat, plastidic ω-3 desaturase gene expression rapidly increases when dark-adapted plants are transferred to white light (Horiguchi *et al*., 1996).

Desaturase are also induced upon pathogen viroid inoculation in tomato plants (Gadea *et al*., 1996) or by pathogen elicitors added to cultured parsley cells (Kirsch *et al*., 1997). The function of antifungal compounds in modulating the level of avocado fruits resistance to *Colletotrichum gloeosporioides* leads us to try to understand the regulation and the possible stages involved in its biosynthesis (Prusky *et al*., 1991; Prusky and Keen, 1995; Ardi *et al*., 1998). The presence of a multiple double bond in the antifungal compound molecules ((Z, Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene and
(Z, Z, E)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5, 12,15-triene)) (Prusky and Keen, 1995; Domergue *et al*., 2000) suggests that desaturation is an essential step in the biosynthesis of diene.

To initiate the study of diene biosynthesis regulation, we cloned stearoyl-ACP desaturase and tested its expression under conditions that enhance the level of antifungal diene (Ardi *et al*., 1998; Leikin-Frenkel and Prusky, 1998) and the resistance of the avocado fruit to *Colletotrichum gloeosporioides.*

Materials and methods

cDNA libraries

Total RNA of ethylene- exposed and non exposed 10-month- old avocado fruits (*Persea americana* Miller var. drymifolia (Schidl. and Cham.) S.F. Blake cv. ‘Fuerte’) was isolated from the mesocarp using TRI REAGENT (Sigma St Louis, Mo). The polyA+ RNA was isolated by using PolyATtract mRNA isolation systems (Promega). Both ethylene exposed and non exposed cDNAs were synthesized by using ZAP express cDNA synthesis kit (Stratagene, USA) and the libraries were packaged in the ZAP express vector (Stratagene, USA) according to the manufacturer’s instructions.

Biotic and abiotic treatments of the avocado fruits

All the treatments were applied on freshly harvested unripe cv. ‘Fuerte’ fruits.

Fruits were exposed to ethylene (40 ml/l), 30% CO_2, challenge inoculation with *Colletotrichum gloeosporioides* isolate Cg-14 and wounding as described by Ardi *et al*., (1998). Low temperature included exposure of fruits to 4 ^oC (22 ^oC as a control) either in the dark (fruits were completely covered), or in the light (exposed to cool white fluorescent light at 140 mmol /m ^{2} sec for up to 24 h).

The effect of shifting from dark to light was examined in fruits exposed for 18 h to darkness at 4 ^oC or 22 ^oC and then shifted to the light or maintained in the dark for another 6 h. Fruits exposed to the different treatments were sampled to determine Δ^9 stearoyl-ACP desaturase transcript level, diene level and decay development. These samples were frozen with liquid nitrogen and stored at –80 ^oC until use.

Fruit resistance to fungal attack and statistical analysis

A single-spore isolate of *C. gloeosporioides* (telemorph: *Glomerella cingulata*), isolate Cg-14, obtained from decayed avocado fruits was used for inoculation experiments (Yakoby *et al*., 2000). The fungus was maintained on Matur's medium (M3S media) at 20 ^oC. Inoculation was carried out on fruits following the different treatments by applying 10 ml of conidial suspension (1.5 x 10^6 conidia /ml) at six inoculation spots on the longitudinal axis of the fruit, three on each side. Following inoculation, fruits were incubated at 22 ^oC, 90% RH for 24 h and then transferred to normal humidity conditions (70-80% RH) at 22 ^oC until fruit ripening and symptom development. Symptoms of decay on pericarp at the artificial inoculation sites, was denoted as dark spots and the lesion diameter expressed in mm. Symptoms of decay in naturally infected fruits was expressed as percentage of the infected area out of whole fruits as described earlier (Prusky *et al*., 1991) out of whole fruits as described earlier (Prusky *et al*., 1991). Inoculation experiments were repeated three times. Standard deviation of the means was calculated, and the differences between means were analyzed by analysis of variance. Presented results are from one representative experiment.

Northern blot analysis

Total RNA was isolated from avocado mesocarp, using a modified phenol/SDS method for plant RNA preparation (Ausbel *et al*., 1992). Samples (0.5 g) were ground with a mortar and pestle under liquid nitrogen and were transferred to 6 ml grinding buffer pH 8.2 (0.18 M Tris-HCl; 0.09 M LiCl; 4.5 mM EDTA pH 8; 1 % SDS) and

mixed. To each sample, 6 ml phenol:chloroform were added and the sample was incubated at 50 ^{o}C for 20 min. The samples were subjected to phenol:chloroform extraction twice more and washed with 6 ml chloroform. The pellets were resuspended in 1 ml of water and precipitated twice with 2 M LiCl (final concentration), resuspended again in water and precipitated with sodium acetate/ethanol following washing with ethanol. RNA from each sample was denatured and separated (fifteen mg of per lane) in 1.1% formaldehyde agarose gels and transferred to Hybond-N membrane as previously described (Madi *et al*., 1993).
The 1.8 kb *Eco*RI-*Xho*I fragment from pPAD1 (avocado cDNA) was prepared by random-primed synthesis. Probes were used in RNA blot analysis at a hybridization temperature of 42 ^{o}C in the presence of 50% formamide. Hybridizing PAD1 RNA was quantified with MacBAS v2.3 software (Fujifilm). To measure loading variations ethidium bromide -stained total RNA was quantified by photographing the gels using the Bio Imaging System (Dinco & Rhenium) and creating an image file using the Liscap software (Dinco & Rhenium). RNA in the different lanes was quantified using MacBAS v2.3 (Fujifilm).

Fatty acid analysis

Total fatty acid analysis was carried out according to Leikin-Frenkel and Brener, (1987) from 1 g of avocado pericarp or mesocarp. Fatty acid methyl esters were analyzed by gas liquid chromatography in Hewlett Packard equipment, built with a capillary column, apiezon-silica (Suopelco), and helium as the carrier gas. The temperature program was isothermic at 190 ^{o}C for 10 min, increasing at 1 ^{o}C per min for another 10 min, under these conditions all the fatty acids from miristate to eicosahexenoic, were separated according to their chain length and number of double bonds. The retention times were compared with those of true standards (Sigma) and the areas of the peaks were integrated. Quantification was also possible due to the incorporation of heptosadecanoic acid as an internal standard in all samples before the methylation step.

Antifungal diene extraction

Avocado pericarp or mesocarp (1-2 g,1- 2-mm deep slices) were homogenized in 95% ethanol in an Omni-Mixer (Sorvall; Dupont Co., Newton, CT.) at full speed for 3 min. The ethanol extract was dried in a rotary evaporator at 40 ^{o}C, brought to 10 ml in

distilled water, and the organic phase was extracted by fractionation with dichloromethane. Following two extractions, the organic phases were pooled, dried with anhydrous $MgSO_4$ (Riedel-deHaen, Seelze, Germany), and evaporated to dryness. Samples were dissolved in 1 ml of ethanol (analytical grade; BioLab, Jerusalem, Israel) and analyzed by high-performance liquid chromatography (Prusky *et al*., 1991). Each experiment was run in four replicates. The diene level was expressed as mg / g of fresh weight (FW). Standard deviation of the means was calculated, and the differences between means were analyzed by analysis of variance.

Incorporation of C^{14} linoeate.

Avocado pericarp (or mesocarp) 1mm thick, was sliced into small pieces (3x1x1mm). Three grams of tissue per sample was incubated with labeled (1-^{14}C) linoleate (NEN, Boston Ma. USA) in 5 ml of 50 mM phosphate buffer pH 7.2 for different length of time (1-24 h) at room temperature. Addition of equal volume of ethanol ended the reaction. At zero time controls the ethanol were added with the labeled linoleate. The non-incorporated linoleate was washed twice with phosphate buffer. Diene extraction was done as described above, and separated by RP-HPLC. The antifungal diene was collected as described before and radioactivity was measured in the presence of Ultima Gold (Packard Inst. Co. Meriden, Ct. USA) in a liquid sentillation counter.

Results

Cloning of a Δ^9 stearoyl-ACP desaturase from avocado

cDNA libraries constructed from ethylene- exposed and nonexposed avocado fruits were screened, using the full - length cDNA clone pMRCD9 of *Ricinus communis* as a probe. As a result of screening $4x10^4$ clones in both libraries, 23 putative clones from the ethylene- exposed cDNA, vs. only five putative clones from the nonexposed cDNA were isolated. Four clones were sequenced and three shared homology to Δ^9 stearoyl-ACP desaturase. One clone, PAD1, a 1818-bp showed 77% homology to the DNA sequence of pRCD1 from *R. communis*. PAD1 contains a 1188-bp open reading frame and encodes a 396-amino- acid protein of 45.4 kDa with 89% amino acid homology and 84% identity to the castor clone pRCD1. PAD1 also shares considerable homology with known Δ^9 stearoyl-ACP desaturases (Thopson *et al*., 1991; Arondel *et al*., 1992; Sato *et al*., 1992; Taylor *et al*., 1992; Cahoon *et al*., 1994). The deduced amino- acid sequence contains two regions of sequence identity to the

two peptides (DETGASP and DYADILE) identified by direct N-terminal sequencing of tryptic fragments derived from the purified avocado stearoyl ACP desaturase identified by Shanklin and Somerville (1992). The alignment of the deduced amino acid sequence of the avocado clone, PAD1 (AF116861), with the desaturases of castor (M59857), cucumber (M59858), *Helianthus* (U91339) and *Brassica* (X74782) (Figure 1) shows the high sequence similarity between desaturases of five distantly related families, suggesting that the sequences are highly conserved among higher plants.

Induction of PAD1 transcript and the antifungal diene by different stimuli.

Inoculation of fruits with *C. gloeosporioides* or exposure to ethylene (40 µl/l), 30% CO_2 or wounding all showed increases of 25% to 50% in the mesocarp antifungal diene over control fruit (Table 1). Diene level in the mesocarp of ethylene-treated fruits was elevated after 3 h of exposure. Incorporation of C^{14}-linoleate into the antifungal diene following exposure to ethylene showed increased 3 h but the level of the C^{14} labeled diene declined 2 h later (Figure 2).

Analysis of the total RNA extracted from the mesocarp revealed that PAD1 transcript increased significantly in all treatments which induced diene level, PAD1 transcript was most abundant after 24 h of treatment, whereas some induction in the control fruits was also observed at 24 to 48 h (Figure 3).

Effect of temperature and light conditions on PAD1 transcription, fatty acids composition and the antifungal diene levels

Exposure of avocado fruit for 6 h to 4 ^{o}C enhanced PAD1 transcription more than at 22 ^{o}C, no matter if the fruits were exposed to light or darkness; however dark treatments induced higher expression than light treatments (Figure 4). If fruits were exposed to a longer darkness period (18 h) at 4 ^{o}C or at 22 ^{o}C PAD1 expression was higher in fruits kept in the dark, no matter the temperature, than in the tissue of fruits transferred to light for another 6 h. (Figure 5).

Fatty acid composition (Figure 6A) in mesocarp of fruits exposed to 4 ^{o}C at dark show that the content of 18:2 and the antifungal diene levels were higher by 33% and 45% respectively than in fruit exposed 22 ^{o}C (Figure 6B).

Effect of temperatures and light/dark conditions on decay development

To examine possible involvement of Δ^9 stearoyl-ACP desaturase in defense response of avocado to *C. gloeosporioides*, we followed the decay development in fruits with elevated or reduced D^9 desaturase expression. Decay development in the artificially (Figure 7A)or naturally (Figure 7B) infected fruits was significantly higher when fruit exposed to light at 22 ^oC Whereas when fruits exposed to dark at 22 ^oC or to light at 4 ^oC the decay was significantly reduced. The lowest level of decay was always observed when fruits were exposed to 4 ^oC in dark (Figure 7A and B).

Discussion

The preformed antifungal (Z, Z)-1-acetoxy-2- hydroxy- 4-oxo- heneicosa-12, 15-diene is involved in avocado resistance to *C. gloeosporioides*. Several elicitors have been reported to induce the increase of this compound (Prusky and Keen, 1995; Ardi *et al*., 1998; Leikin-Frenkel and Prusky 1998) however the biosynthetic pathway involved was never described. We have assumed that synthesis of the diene involves extension of a linoleic acid starting compound by conventional two-carbon elongation. As a first stage to determine the biosynthetic pathway involved in the synthesis of the antifungal diene we have found that Δ^9 stearoyl-ACP desaturase cloned from avocado fruits is significantly expressed by similar elicitors that induce the antifungal diene, ethylene (40 μg/l), CO2 (30%) wounding, *C. gloeosporioides* inoculation and cold treatment (4 ^oC). Elevated transcription of desaturases (ω-3, $\mathbf{\Delta}^2$ and $\mathbf{\Delta}^9$) at low temperature had been reported in plants (Miquel *et al*., 1993; Somerville, 1995; Young Moon *et al*., 1995; Ishizaki-Nishizawa *et al*., 1996) and was implied to be required for resistance to chilling by altering the lipids composition in the membrane (Miquel *et al*., 1993). In avocado, the increase in $\mathbf{\Delta}^9$ stearoyl-ACP desaturase transcripts level in fruits exposed to

4 ^0C compared to fruits exposed to 22 ^0C, suggest that avocado fruit responds to low temperature in the same manner like other plants by increasing the D^9 stearoyl-ACP desaturase transcription level and the unsaturated fatty acids level (mainly linoleic acid). However, no report has been published on induction of $\mathbf{\Delta}^9$ stearoyl-ACP desaturase in correlate with the increase of antifungal diene synthesis and its effect on fungal resistance.

Another elicitor that was found to activate the PAD1 is storage under darkness. In *A. thaliana FAD7* expression and the wheat plastidic ω-3 desaturase transcripts

rapidly increased when dark-adapted plants were transferred to white light (Horiguchi *et al*., 1996). In avocado however, Δ^{9} stearoyl-ACP desaturase expression level increased in fruits exposed to darkness compared to fruits exposed to light. The effect of light on the transcription level is more pronounced when fruits exposed to darkness were transferred to light. In this case the level of transcription was markedly reduced independently of the temperature that fruits were exposed.

The concurrent increase in Δ^{9} stearoyl-ACP desaturase transcription, 18:2 content, and the incorporation to C^{14} into the antifungal diene suggest the Δ^{9}- stearoyl-ACP desaturase is involved in the diene biosynthesis probably by increasing the level of the diene precursors, that can incorporate into the antifungal diene. This increase in diene level significantly affect fruit susceptibility to *C. gloeosporioides* attack, as observed in fruits exposed to 4 ^{o}C at darkness. Alternatively induction of desaturases (Gaeda *et al*., 1996; kirsch *et al*., 1997) and lipids desaturation was suggested to be an early component of the complex of defense response (Kirsch *et al*.,1997). Acquired resistance to fungal attack mediated by unsaturated lipids as a result of increased activity of desaturase had been reported so far only by Wang *et al*., (1999), where it was shown that expression of $\mathbf{\Delta}^{9}$ stearoyl-ACP desaturase of yeasts in tomato plants enhanced its resistance to powdery mildew by elevating desaturated fatty acids. Cohen *et al.*, (1991) had reported that external treatment of plant leaves with unsaturated fatty acids induced systemic resistance in potato plant against *Phytophthora infestans.* Unsaturated fatty acids can transform to lipids peroxides that can acts as antimicrobial compounds Wang *et al*., (1999), or by stimulating the signal transduction pathway via octadecanoid pathway to signal molecules such as jasmonate (Vick and Zimmermann, 1984; Tzeng and DeVay, 1993). Modulation of resistance by ROS induced in unripe avocado fruits by fungal infection was suggested by Beno-Moualem and Prusky (2000).

Present results indicate that elicitors activate Δ^{9} stearoyl-ACP desaturase transcript expression, linoleic acid and antifungal diene content (Prusky *et al*., 1991; Prusky and Keen , 1995). Although our data does not directly support the involvement of desaturase in diene synthesis, the data presented suggest that desaturase is an important step in diene synthesis.

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Legends to figures

*Figure 1***.** Comparison of the deduced amino acid sequence of the avocado homologe (PAD1) encoding Δ^9 stearoyl-ACP desaturase and members of the Δ^9-desaturase family. The entire coding sequence of PAD1 was compared with the *Ricinus communis* (castor), *Cucumis sativus* (cucumber), *Hlianthus annuus* and *brassica napus.* Identical or conserved residues are boxed.

Figure 2. Incorporation of C^{14}-linoleate into the antifungal diene in the presence of ethylene treated mesocarp fruit tissue.

Figure 3. Expression of PAD1 encoding Δ^9 stearoyl-ACP desaturase in response to exposure to CO_2, ethylene, wounding and inoculation with *C. gloeosporioides.* RNA was extracted (20 mg) from the mesocarp of fruits, electrophoresed and probed with *Xho*I-*Eco*RI fragment of PAD1, the avocado Δ^9 stearoyl-ACP desaturase. (EtBr-Ethidum bromide)

Figure 4. Expression of PAD1, Δ^9 stearoyl-ACP desaturase in response to light and low temperature. (A), Freshly harvested fruits were exposed for 6 h to dark and light

conditions at 4 ^{0}C and 22 ^{0}C. After the exposure period RNA was extracted (20 mg) from the mesocarp of fruits, electrophoresed and probed with *Xho*I-*EcoR*I fragment of PAD1, the avocado Δ^9 stearoyl-ACP desaturase. (B) Quantification of hybridizing RNA is shown as percentage of maximum measured photostimulated luminescence (PSL). Correction for loading variation was done as described in Material and methods.

Figure 5. Expression of PAD1, Δ^9 stearoyl-ACP desaturase in fruits pre-adapted to darkness, in response to light and low temperature (A), Freshly harvested fruits were adapted to darkness for 18 h at either 4 ^{0}C or 22 ^{0}C and then transferred to light for 6 h. After the exposure, total RNA was extracted from the mesocarp and subjected to Northern blot analysis (as described in Figure 2).

Figure 6. Linoleic acid composition and antifungal diene levels in mesocarp of fruit exposed for 6 h to dark at 4 ^{0}C and 22 ^{0}C.

Figure 7. (A), Decay development in fruits pre-exposed for 24 h to light at 22 ^{0}C (◊), Light at 4 ^{0}C (▲), Dark at 22 ^{0}C (■) or Dark at 4 ^{0}C (x) and then artificially inoculated with *C. gloeosporioides*. (B), Decay development in naturally infected fruit pre -exposed to the same treatments as in A.

Table 1. Effect of exposure to ethylene, CO_2, wounding and inoculation on diene level

	Diene level (mg/g.F.W.)

	Pericarp	Mesocarp
Control	646+35	1200+67
Ethylene*	1030+54	1600+30
CO_2	1310+165	1834+145
Wounding	1150+30	1750+50
Inoculation	960+57	1647+65

*The diene level as determined at 3 h after exposure ethylene, otherwise after 48 h.

3. PAPERS IN PREPARATION

Isolation of a cDNA Clone Encoding an Avocado (*Persea Americana*) Δ^{12}-Fatty Acid Desaturase (accession number: AY057406)

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The primary products of fatty acid synthesis are palmitoyl-acyl-carrier (16:0-ACP) and stearoyl-ACP (18:0-ACP). The soluble plastid enzyme, stearoyl-ACP desaturase (EC1.14.99.6), introduces the first double bond into stearoyl-ACP, between carbons 9 and 10, to produce oleoyl-ACP (18:1 Δ^9-ACP). Additional double bonds are introduced into oleic acid or palmitic acid by Δ^{12}-desaturases after incorporation of the fatty acids into lipids (Shanklin and Somerville, 1991; Thompson et al., 1991). Oleate (18:1) produced by stearoyl-ACP Δ^9-desaturase must be sequentially desaturated by Δ^{12} and Δ^{15}-desaturase to form the major fatty acid of photosynthetic tissues, α-linolenate (18:3) (Harwood, 1997). Microsomal Δ^{12}-desaturases transform oleic acid into linoleic acid preferably from exogenous oleoyl-CoA rather than elaidoyl-CoA (Lomascolo, et al., 1996).

It was hypothesised that Δ^{12}-fatty acid desaturases (EC1.14.99.-) may catalize the desaturation of the antifungal diene (Prusky and Keen, 1993; 1995; Prusky et al., 1991). Diene is enhanced by ethylene (Leikin-Frenkel and Prusky, 1998), CO_2, wounding or inoculation of avocado fruits with *C. Gloeosporioides* (Ardi et al., 1998).

Table 1. Characterization of Δ^{12}-desaturase cDNA from avocado (*Persea americana*)

Organism:
Avocado (*Persea americana* cv Fuerte).

Gene Product:

Δ^{12}-desaturase (EC1.14.99.-).

Function:

Introduces the second double bond into oleic acid ($18:1^{\Delta 9,trans}$) between carbon 12 and 13 to produce linoleic acid ($18:2^{\Delta 9,12}$).

Techniques:

Five different avocado genomic DNA fragments corresponding by sequence to Δ^{12}-desaturase were cloned by PCR with two degenerate primers, C A Y G A R T G Y G G N C A Y C A Y (upstream) and G C Y T T N K T N G C Y T C C A T (downstream) (synthesized by Life Technologies Ltd. UK), designed according to two boxes of conserved amino acid sequences, H E C G H H (upstream) and M E A t K A (downstream), of Δ^{12}-desaturases in higher plants. These fragments did not contain intron and were 692-bp long. One of the five DNA fragments served as probe to screen an avocado fruit cDNA library (constructed by L. Madi). An 867-bp cDNA clone with stop-codon and poly-A was obtained from the library. 5'-RACE PCR permitted cloning of the missed 5'-fragment, which contained 282-bp of cDNA with start-codon and 5-UTR sequences. Assembly of these two cDNAs generated a cDNA sequence containing the entire putative ORF of avocado Δ^{12}-desaturase with 3-UTR, poly-A and 36-bp 5-UTR.

Characteristics of cDNA:

The cDNA was 1250 bps long and encoded 382 amino in an open reading frame. Comparison of deduced amino acid sequence with *Arabidopsis thaliana* Δ^{12}-desaturase showed 83.8 % identity.

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Isolation of a cDNA Clone Encoding An Serine/threonine Kinase (accession number: AY057407) from Avocado Fruit (Persea americana)

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Enzymes of the eukaryotic protein kinase superfamily catalyze the reversible transfer of the γ-phosphate from ATP to amino acid side chains of proteins. In plants, protein phosphorylation has been implicated in responses to many signals, including light, pathogen invasion, hormones, temperature stress, and nutrient deprivation (Stone and Walker, 1995). Studies have implicated protein phosphorylation in the expression of disease resistance and self-incompatibility (Dietrich et al., 1990; Wang et al., 1996).

Serine/threonine kinases (EC 2.7.1.-) have been implicated as key elements in the signaling processes in plants (Sheen, 1996). They are found in various subcellular locations, which suggest that this family of kinases may be involved in multiple signaling pathways and the network of protein serine/threonine kinases in plant cells has been refered as a “central processor unit” (Hardie, 1999).

A fragment corresponding to serine/threonine kinase was cloned from an avocado substract cDNA library that was constructed under conditions of antifungal diene induction (Prusky and Keen, 1993; 1995; Prusky et al., 1991). The preliminary results indicted that this serine/threonine kinase might be induced under conditions (ethylene treatment) that induce antifungal diene synthesis.

Table 1. Characterization of serine/threonine kinase cDNA from avocado (*Persea americana*)

Organism:
Avocado (*Persea americana* cv Fuerte).

Gene Product:
Serine/threonine kinase (EC 2.7.1.-).

Function:
Serine/threonine kinase catalyze the reversible transfer of the γ-phosphate from ATP to serine/threonine amino acid side chains of proteins (Stone and Walker, 1995).

Techniques:
A 183-bp cDNA fragment corresponding to serine/threonine kinase was identified among cDNA clones from an avocado subtraction cDNA library that was constructed under conditions of antifungal diene induction. The preliminary results indicted that its mRNA transcription was induced under conditions that induce the antifungal diene synthesis. The DNA sequence information served to design primers for 3'-RACE PCR with 5'/3'-RACE PCR Kit following manufacturer's instruction, since the attempts to isolate the cDNA clone from avocado cDNA library failed. A total 337-bp sequence contained 165-bp DNA deducing the C-terminal protein of the putative protein, 3-UTR and poly-A was cloned by 3'-RACE PCR. An 896-bp fragment overlapping the 5-region of the 183-bp fragment was cloned from a mini genomic DNA library of avocado. No start codon and no any intron existed in this 896-bp fragment. A 5'-RACE PCR fragment of 206-bp long including 118-bp codoning region containing start codon that deducing N-terminal of the putative serine/threonine kinase protein, and 88-bp 5-UTR sequence was cloned. Assembly of these four fragments created a 1622-bp long of putative avocado serine/threonine kinase with 5-UTR, 3-UTR and poly-A tail.

Characteristics of cDNA:

The total size of the assembled serine/threonine kinase of avocado was 1622-bp long encoding a putative ORF of 453 amino acids and 88-bp of the 5-UTR and 172-bp of the 3-UTR including poly-A tail. The alignment of deduced 453 amino acid with serine/threonine kinase of *Arabidopsis thaliana* resulted 67.3% identity.

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Cloning and Expression of Fatty Acid Elongase from Avocado Related to Biosynthesis of Antifungal Diene

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Very-long-chain fatty acids (VLCFAs) have chain with 20 to 30 carbons, or more. The major site of VLCFA synthesis is the epidermal cells where they are utilized for the production of waxes embedded in the suberin and cutin and the epicuticular waxes which cover the aerial surfaces of the plant (Post-Beittenmiller, 1996). VLCFAs are synthesized by a microsomal fatty acid elongation (FAE) system by sequential additions of C2 moieties from malonyl coenzyme A (CoA) to pre-existing C18 fatty acids derived from the *de novo* fatty acid synthesis (FAS) pathway of the plastid (Fehling and Mukherjee, 1991; Fehling et al., 1992).

In *Arabidopsis*, mutations in the FAE1 gene result in highly reduced level of seed VLCFAs (James and Dooner 1990; Lemieux et al., 1990) and in a deficiency in acyl chain elongation activities from C_{18} to C_{22} and C_{20} to C_{22}, suggesting that the product of the FAE1 gene is required for both elongation steps (Kunst et al., 1992). FAE1 is the rate-limiting enzyme for VLCFAs biosynthesis in *Arabidopsis* seed (Anthony and Ljerka, 1997).

It was hypothesized that fatty acid elongases may catalyze the carbon-chain elongation of preformed antifungal compound, 1-acetoxy-hydroxy-4-oxo-heneicosa-12, 15 diene in avocado fruits (*Persea americana*) (Prusky, 1996; Prusky and Keen, 1993; Prusky et al., 1982, 1983, 1985, 1991, 1994). Diene is enhanced by ethylene (Leikin-Frenkel and Prusky, 1998), CO_2, wounding or inoculation of avocado fruits with *C. gloeosporioides* (Ardi et al., 1998).

Cloning of Fatty Acid Elongase Genomic Fragments

Two avocado genomic DNA fragments of 226-bp long were cloned using the degenerative primers, CAYTTYTGYATHCAYGC (upstream) and SWRTTRCAYTTRAANCC (downstream). The primers were designed based in two conserved amino acids sequences HFCIHA (upstream) and GFKCNS (down stream) of KCS1 synthase from *Arabidopsis* (KCS1, AF053345), FAE1 KCS from *Arabidopsis* (FAE1, U29142), FAE KCS from *Brassica napus* (*B. napus*, U50771) and FAE KCS from jojoba (jojoba, U37088). These two genomic DNA sequences

code for 75 amino acids that differ only in 2 amino acids and no intron was found.

Based on the sequence information at their 5' end, we PCR cloned a 799-bp genomic DNA fragment from a mini genomic DNA library of avocado. This genomic sequence contained 441-bp exon at its 3' end and 358-bp intron at its 5' end.

Based on the same sequence information at of the 3' ends, we used 3'-RACE PCR and cloned a 333-bp fragment containing poly-A. It had 114-bp coding area and 219-bp 3'-UTR with poly-A.

Assembly of these three fragments created an 1141-bp sequence of the putative FAE of avocado. The amino acid alignment of the assembled sequence with FAE1 (U29142) of *Arabidopsis thaliana* shows high homology and 68.2% identity.

The expression of that gene during the elicitation of the antifungal diene is presently being studied.

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1		1	2
Submitted, in review, in preparation			3	3
Invited review papers				
Book chapters			4	4
Books				
Master theses				
Ph.D. theses			1	1
Abstracts			1	1

Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Lea madi,

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	3	5
Longer Visits (Sabbaticals)		1		1

Description of Cooperation:
Cooperations was based on the daily consultation through the e-mail and Meetings in international conferences. Dr. Prusky spent more than 2 months in Dr. Browse lab working and developing different aspects of the project

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				